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Towards the objective of developing site-specific antagonists to tetrodotoxin (TTX) and saxitoxin (STX), work has been progressing in two directions. First, three new TTX analogues (6-epi TTX, 11-deoxyTTX and chiriquitoxin) and two new STX analogues (deoxydecarbamoylSTX and decarbamoylneoSTX) have been studied for their relative potencies in blocking the sodium channel. From these studies, the number of stereospecific similar groups in TTX and STX have been expanded from 3 to 5. As a result of these efforts, the TTX/STX binding site has been deduced as being a "cave" of about 8 Å wide, 6 Å tall, and 4-5 Å deep. A second direction of our work is synthesis of site-specific antagonists. Because of the new understanding of the TTX/STX binding site, new chemical directions for synthesis of antagonists are under way. Additionally, a new chemically reactive TTX analogue, 11-oxo TTX, has been made synthetically, which will serve as a most useful intermediary for further derivatization.

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SITE-SPECIFIC ANTAGONISTS TO TETRODOTOXIN AND SAXITOXIN

Annual Report

C. Y. Kao

May 1, 1990

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7094

State University of New York, Downstate Medical Center
Brooklyn, NY 11203-9967

INTRODUCTION

Project goals. The objective of this project is to generate more knowledge about the specific chemical structure of the tetrodotoxin (TTX)/saxitoxin (STX) binding site on the voltage-gated sodium channel protein. It is hoped that from such knowledge, site-specific antagonists to these toxins can be developed rationally. Additionally, identification of the binding site will greatly aid further understanding of the three-dimensional structure of the sodium-channel, and such knowledge will facilitate our understanding of the actions of other sodium-channel effectors, and the development of appropriate specific antagonists.

The project has been progressing on two parallel tracks: (a) to expand and refine current knowledge of the structure-activity relations of TTX/STX analogues, and (b) to produce new synthetic compounds which might mimic or block the actions of TTX/STX by interacting with the TTX/STX binding site. On track (a), the work consists largely of electrophysiological studies of newly discovered natural analogues of TTX and/or STX, utilizing the voltage-clamped preparation to study specific ionic conductances. This phase of the project is now nearly complete, because new data obtained during the past year have led us to identify almost all of the stereospecific similar surface groups of the TTX and STX molecules. By complementarity considerations, we have derived the approximate shape and dimensions of the TTX/STX binding site.

On track (b), past attempts to synthesize new compounds have been hampered by the limited knowledge of potential reactive binding sites. Although new cyclic guanidinium compounds have been synthesized, unforeseen technical difficulties have prevented their full isolation for testing purposes. However, because of new developments in track (a), we have new views as to the type of molecules that could conceivably interact with the TTX/STX binding site, and are taking new directions in these synthetic efforts. More significantly, a reactive TTX derivative has been produced with reasonable yield, which will permit the synthesis of specific marker compounds for locating the TTX binding site.

Background. Tetrodotoxin (TTX) and saxitoxin (STX) are important neurobiological tools because of their specific reaction with the voltage-gated sodium channel. They are among the most lethal, low molecular-weight, non-protein toxins known, each with a LD_{50} of 10 ug/kg body weight. They are very different in chemical structure, but their biological actions are virtually identical.

Although they came into use in the 1960's, the chemical basis of their action remains unclear. The difficulty is attributed chiefly to obstacles in studying the chemical properties of these molecules. For example, TTX is practically insoluble in any solvent except in slightly acidified water. Early attempts at modifying the structures generally led to marked loss of biological activity. For STX, uncertainties about its structure persisted into the early 1970's. However, because of advances in separation technology, several natural analogues of both TTX and STX were discovered in the mid 1970's. Most of these analogues turned out to have some biological activity, as the chemical modifications were often small. Renewed studies on the structure-activity relations of TTX and STX were then initiated. On the basis of those studies, some active groups in each toxin molecule were recognized. Significantly, three centers were identified in each toxin molecule that showed very close stereospecific similarities

(see summary in reference 1). These groups are:

TTX	STX
1,2,3 guanidinium	7,8,9 guanidinium
C-9, hydroxyl	C-12 hydroxyls (gem-diol)
C-10 hydroxyl	

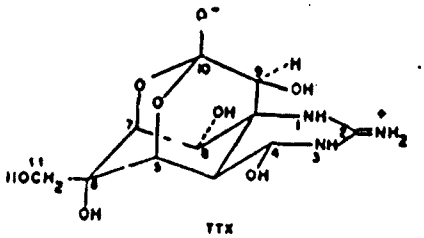
These groups form the basic active portions of each toxin molecule. From this information, we attempt to fan out in search of refinements and other important details which may add to the success of this project.

During the past year, we have identified two new stereospecific similar groups in each toxin molecule, and a third potentially reactive group in STX. Therefore, almost all the surface active groups in each molecule are identified. Consequently, we are now able to describe, for the first time, the rough shape and dimensions of the TTX/STX binding site. Because of this new development, we have formed some new views as to the type of molecule which could potentially occupy the site, and are now persuing this line of approach. Additionally, to further support our position, we have produced a reactive derivative of TTX which will serve as an intermediary for making markers for the TTX/STX binding site. We anticipate that with such markers in hand, we should be able to locate and identify the peptide residues of the binding site.

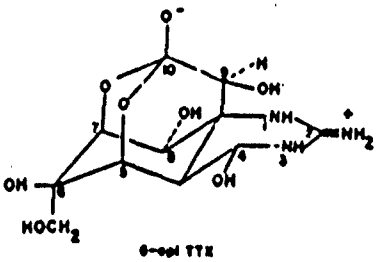
WORK DONE IN THE PAST YEAR

Electrophysiological Work

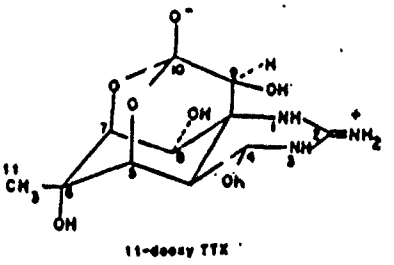
6-epi TTX and 11-deoxy TTX. These compounds are natural analogues of TTX, discovered by Yasumoto and his collegues in an Okinawan newt, Cynops ensicuda (2). Their structures are shown below. I have reported on the results of our studies of their biological actions in previous quarterly reports and the annual report of May 1, 1989. However, I will briefly restate the main points in order to facilitate an appreciation of the more recent conclusion with regard to active portions of the entire TTX molecule.



TTX



6-epi TTX

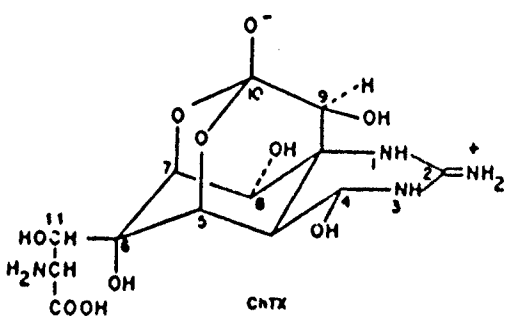


11-deoxy TTX

6-epi TTX differs from TTX only in having the groups on C-6 and C-11 transposed. Yet, it is only 3% as active as TTX in blocking the sodium-channel. 11-deoxy TTX differs from TTX only in having the hydroxymethyl function ($-CH_2OH$) on C-11 replaced by a methyl group (CH_3). Its sodium-channel blocking activity is about 1% that of TTX (3).

With an equilibrium dissociation constant (K_d) of 3.4 nM, the binding energy of TTX to its receptor site (Gibbs free energy change, ΔG) is in the range of 48 kJ. From the experimentally determined K_d for 6-epi TTX and 11-deoxy TTX, the ΔG for 6-epi TTX is 40 kJ, and that for 11-deoxy TTX is 36 kJ. These differences in ΔG are consistent with the loss of 1 to 2 hydrogen-bonds. Therefore, my view is that the -OH group in the hydroxymethyl function of C-11 forms an important hydrogen-bond with some H-acceptor group in the receptor site. In the case of 6-epi TTX, the epimeric configuration alters the steric position of the C-6 -OH and the C-11 -OH to weaken the H-bonds that would normally exist for TTX. These are the first clear and objective evidence that -OH groups at the C-6 end of the TTX molecule are actually involved in binding, because up until these results, existing evidence tended to suggest otherwise.

Chiriquitoxin (ChTX). This is a natural analogue of TTX, first discovered in 1973 by Kim, Brown, Mosher and Fuhrman (4) in a Costa Rican frog, **Atelopus chiriquensis.** Assays on mice lethality tests, and on sodium-channel blockade showed it to be equipotent as TTX (1, Kao et al., ChTX). Until then, no analogue of TTX had been known to have any significant biological activity. Therefore, its discovery propelled the renewed interest in studies of possible structure-activity relations of TTX. Studies on ChTX were difficult because of the very limited amount of material first available, and the structure was not determined. Further collection of raw material was unsuccessful, chiefly because of climatic aberrations in Central America. In 1988, with funding from an NIH grant, a new batch of skins from A. chiriquensis was collected. Now, in collaboration with Prof. Yasumoto and Dr. Mari Yotsu, about 12 mg of ChTX were isolated. Based on about 6 mg of this new material, the structure has now been determined:

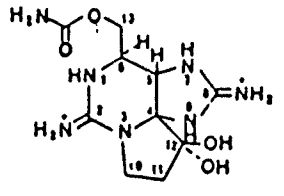


ChTX

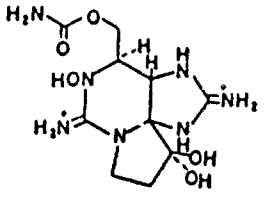
It can be seen that all the stuctural features of TTX are present in ChTX, but the latter has in addition a glycine moeity attached at the C-11 function. Renewed tests using an improved voltage-clamp method on skeletal muscle fiber showed that the ED_{50} for blocking the sodium channel is identical with that for TTX (5, 6). The only differences from TTX are seen in the different pH-dependence of the potency. Such differences are relatively minor, and are attributable to the influence of the terminal glycine attachment. What is most significant is that whereas minor alterations in structure such as seen in 6-epi TTX and 11-deoxy TTX produced marked loss of activity, a rather bulky addition as in ChTX produced no effect at all. This observation contributes to the conclusion (see below) on the possible conformation by which TTX and STX exert their

sodium-channel blockade.

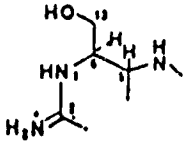
Deoxydecarbamoylsaxitoxin (DeoxydcSTX) and decarbamoylneosaxitoxin (dcneoSTX). These natural analogs were isolated by Dr. Y. Oshima of Prof. Yasumoto's department in Tohoku University, Sendai. DeoxydcSTX was isolated from Mytilus edulis contaminated with Gymnodinium catenatum at Tasmania, Australia. DcneoSTX was isolated from Spondylus sp. contaminated with Pyrodinium bahamense var. compressa at Palau. To explain the significance of these structural variation, we need to recall that one of the only two synthetically modified derivatives of STX is decarbamoylSTX (dcSTX). It could be produced from STX by hydrolysis with concentrated HCl, and the resultant dcSTX retained a good deal (0.2) of the sodium-channel blocking effect of STX (Koehn et al.).



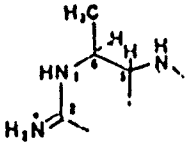
STX



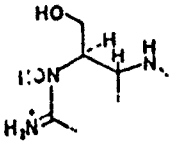
neoSTX



dcSTX



deoxydcSTX



dcneoSTX

An examination of the structures above will show that in dcSTX, there is an -OH on C-13. I have always suspected that that -OH could participate in H-bonding, but had no way to prove it. In deoxydcSTX, the C-13 alcohol function has been changed to a methyl group which cannot form H-bonds. Not surprisingly, the ED_{50} of deoxydcSTX is 618 nM (7; compared with 5.1 nM for STX, and an estimated 25 nM for dcSTX).

NeoSTX differs from STX only in having an -OH on N-1 instead of the -H in STX. It is a problematical compound, because the neoSTX prepared in differed laboratories have widely different specific potencies. In mouse lethality assays, the specific potency differs by 2 to 3 fold; on sodium-channel function of isolated nerves and muscles, the difference could be even more. The 1,2,3 guanidinium function in STX has a pKa of 11, and within the physiological pH range is almost entirely protonated. So, its role in binding to receptor could not be tested. In neoSTX, the -OH on N-1 has a pKa of 6.75, and provides a good opportunity for testing any possible role of the 1,2,3 function in binding to receptor. We found that neoSTX is most potent at pH 6.5, on the acid side of the pKa, and very weak at pH 8.25. These observations suggests that when N-1 -OH is protonated and not charged, it can form H-bond with some acceptor group in the receptor,

possibly a anionic carboxylate function. When the N-1 -OH is deprotonated at pH 8.25, then the negatively charged -O is repelled by that same group in the receptor. On the question of the relative potencies of neoSTX and STX, unfortunately because of the marked variability of the specific potency of neoSTX, I have no definitive conclusion. Nevertheless, the pH dependence of the potency of neoSTX and our interpretation means that the 1,2,3 guanidinium group does have an influence in binding.

Decarbamoylneo STX is significant, because the effect of pH on its potency are the same as in neoSTX (7). This consistency strengthens my interpretation above. Thus, we can assign a point of attachment which is on the opposite side of the C-12 gem-diols. With this assignment, then the STX binding site can only be in a fold of the sodium-channel protein, rather than being on a planar portion of the protein surface.

Probable shape and size of the TTX/STX binding site. Whereas we started this contract year with a general knowledge of 3 active groups each in the TTX and STX molecules, we conclude the year with 2 additional groups for each. When the two molecules are superimposed, such that the active guanidinium group and the C-9 and C-10 hydroxyls in TTX and the C-12 gem-diols in STX are aligned, the C-13 -OH of deSTX corresponds in space with the C-6 -OH of TTX, and the carbonyl in the carbamoyl function of STX corresponds very closely in space with the C-11 hydroxymethyl of TTX. Thus, the corresponding active groups of the two toxin molecules are:

TTX	STX
1,2,3 guanidinium	7,8,9 guandinium
C-9, -OH	C-12 -OH's (gem-diol)
C-10 -OH	
C-6 -OH	C-13 -OH (in deSTX)
C-11 -OH	C-14 carbonyl
---	N-1 -OH (neoSTX)
C-12 groups (glycine in ChTX)	C-11 groups ($-OSO_3$ in gonyautoxins)

Such coincidence suggests that the TTX/STX binding site is in a fold of the protein that surrounds all sides of the TTX or the STX molecule, except one. It is probably 8 Å wide, 6 Å tall, and 4-5 Å deep. The free side is the "enterance to the cave", and that is where the glycine moeity of ChTX sticks out. Therefore, the explanation of why ChTX is equipotent with TTX is that ChTX possesses all the structural features of TTX necessary to bind to receptive groups in the walls of the "cave", and that the glycine group protrudes out of the "enterance" without coming close to any functional groups in the channel protein.

For the same reason, the absence of any serious effects of the large and charged sulphate groups on C-11 in several gonyautoxins in the PSP family is that these sulphate groups probably project out of the "enterance" and are not close to any reactive groups in the binding site. In this view, the very weakness of the 21 sulfocarbamoyl group of PSP toxins is explanable by the steric hinderance of the sulfocarbamoyl tail which prevents the main molecule from entering into the "cave".

Chemical Work

In this area, we have followed two routes. One is to synthesize new types of compounds suitable for reacting with the TTX/STX binding site. The other is to modify the TTX or STX molecule for derivatization. We have worked only with TTX and not with STX, because only TTX is available. We do not have sufficient STX for chemical work.

Previously, when we had only a partial view of the binding site, we focussed our synthetic efforts on making cyclic guanidinium compounds that would have two hydroxyl functions close-by, stereochemically positioned in a manner closely resembling the active portions of the TTX and/or STX molecules. As Prof. H. S. Mosher has repeatedly pointed out, these configurations can be intrinsically unstable because of intramolecular rearrangements. Our expanded view of the binding site opened a wider perspective as to potential types of molecules that could bind to the site. On the basis of this premise, Dr. B. Q. Wu, Senior Research Associate, has planned a number of possible strategies towards synthesizing classes of compounds which woud be worth testing. These efforts have passed beyond the planning stage, but they have not yet been put into the synthetic stage because we are fully engaged at the present time in making a reactive derivative of TTX.

In their continuing investigations of the biosynthetic mechanism and fate of TTX in the animal body, Prof. Yasumoto and some of his colleagues discovered a new derivative of TTX in a south Pacific puffer fish, Arothron nigropuntatus. The compound, called 11-oxo TTX is a C-11 aldehyde in a hydrated form. In the NMR, they also showed that 11-oxo TTX can be reduced to TTX. These important observations suggest that 11-oxo could be a useful intermediary for helping us accomplish the objectives of this contract. For this reason, Yasumoto now holds a subcontract to provide us with some 11-oxo TTX from his isolations.

In the meantime, I feel that we should try to produce some 11-oxo TTX synthetically, to supplement any amount that Yasumoto could provide from natural sources. Using procedures in the open literature, but laboriously modifying the reaction conditions, Dr. Wu has now succeeded in oxidizing TTX into 11-oxo TTX at about 20% yield. We consider this outcome extremely heartening, and are now fully engaged in scaling up the reaction into preparative scales. Our target is to collect 20 - 25 mg of 11-oxo TTX, for derivatization purposes.

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U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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